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Coinfection of Individual Leukocytes With Human Cytomegalovirus and Human Immunodeficiency Virus Is a Rare Event In Vivo

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Infection with the human cytomegalovirus (HCMV) accelerates disease progression in human immunodeficiency virus 1 (HIV-1)-infected individuals. This has been attributed to the transaction of HIV-1 gene expression by HCMV gene products. For transactivation to be effective in vivo both viruses must be present in the same cell. We therefore examined blood samples from 13 HIV-1-infected patients with HCMV viremia for coinfection of individual leukocytes. In four of the patients lymph nodes were also examined. Multiple samples contained defined numbers (between 10 and 1000) of $CD4^+$ lymphocytes or $CD14^+$ monocytes were sorted by a FACS-based automated cell deposition unit. Samples were then analysed by a multiplex nested polymerase chain reaction, which can detect simultaneously HCMV and HIV DNA. The percentage of infected cells was calculated for each virus using the Poisson distribution. Between 0.43% and 6.2% of the $CD4^+$ lymphocytes were infected with HIV and less than 0.15% with HCMV. The level of infection in $CD14^+$ monocytes was always $\leq$0.11% for HIV and ranged between $<$0.05% and 0.58% for HCMV. Only seven of 1030 sorted samples from blood were positive for both viruses. In lymph nodes, none of the 144 samples tested were double-positive. This clearly shows that coinfection of individual human leukocytes with HIV and HCMV is a very rare event in vivo. Therefore, direct transactivation of HIV by HCMV in coinfected cells obtained from blood and lymph nodes may not explain the effect of HCMV on the prognosis of HIV-infected individuals.

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KEY WORDS: HIV, HCMV, monocytes, $CD4^+$ lymphocytes

INTRODUCTION

The incubation period for the acquired immunodeficiency syndrome (AIDS) is variable, ranging from 1 year to more than 10 years. The precise pathogenic mechanisms, which induce progression from asymptomatic infection to overt AIDS are unknown, but multiple factors are likely to be involved [Schnittman and Fauci, 1994].

HIV-infected individuals suffer frequently from opportunistic infections with human cytomegalovirus (HCMV) and other herpesviruses [Macher et al., 1983; Quinnan et al., 1984]. Webster [1991] investigated the role of HCMV in HIV disease progression and showed that HCMV-seropositive patients developed AIDS more rapidly than HCMV-seronegative individuals. Different mechanisms by which HCMV accelerates disease progression have been considered [for review see Webster et al., 1989, 1991].

In vitro studies indicate that intracellular interactions between the two viruses may be involved. Coinfection with HCMV enhances HIV-replication in cell lines [Skolnik et al., 1988]. HCMV like many other viruses such as herpes simplex virus, Epstein-Barr virus, human herpes virus 6, and hepatitis B virus can transactivate the HIV long terminal repeat (LTR). The HCMV immediate early gene products as well as cellular factors were found to be involved in HCMV-mediated HIV transactivation [Davis et al., 1987; Biegalke and Geballe, 1991; Walker et al., 1992; Barry et al., 1991]. One of the mechanisms for transactivation suggested by Webster [1991] is that HCMV immediate early gene products induce cellular transcription factors such as NF-kappa B and SP1, which are known to bind to promoter sequences in the HIV LTR [Jones et al., 1986; Nabel and Baltimore, 1987] and therefore enhance HIV transcription. In further studies, the target sequences for HCMV-mediated transactivation were then localised to TAR, SP1, or the 10-bp element in the TATA region [Walker et al., 1992].

If this transactivation by HCMV is to have a substantial impact on the viral load for HIV in vivo, both viruses must coinfect a significant portion of cells. In fact, coinfection with HIV and HCMV has been demonstrated in

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TABLE I. Patients Examined in the Present Study

Patient	Sex	$CD4^+$ Cells/μl	CDC-stage[b]	HCMV-associated disease
1	f	31	C3	HCMV retinitis[a]
2	f	48	C3	HCMV pneumonia[a]
3	m	30	C3	
4	m	398	B2	
5	m	20	C3	HCMV gastritis[a]
6	m	a: 70	C3	
		b: 28	C3	
7	m	15	C3	
8	m	3	C3	HCMV retinitis[a]
9	f	3	C3	HCMV encephalitis[a]
10	m	400	A1	
11	m	540	A1	
12	m	530	C1	
13	m	130	C3	

[a]The patient suffered from HCMV-associated disease at the time the samples were analysed.
[b]CDC-Classification 1993 [CDC, MMWR, 1993].

the brain and retina [Nelson et al., 1988; Rummelt et al., 1994]. Here HCMV contributes most likely to the pathology observed in AIDS patients [Wiley and Nelson, 1988], but the major reservoir for HIV are the $CD4^+$ lymphocytes from lymph node and blood [Embretson et al., 1993; Schnittman et al., 1989]. However, HIV-infection is also found in the monocyte/macrophage population [Hufert et al., 1989, 1993]. In consequence, coinfection of a significant portion of $CD4^+$ lymphocytes or possibly monocytes is required for HCMV to influence substantially HIV replication by direct transactivation on single cell level.

We, examined therefore, these cell populations in blood and lymph nodes of HIV-infected individuals. Coinfection of individual cells with HCMV and HIV was found to be rare. The intracellular interactions between HIV and HCMV found in vitro are, therefore, most likely not responsible for the more rapid development of AIDS in HCMV-seropositive patients.

MATERIALS AND METHODS

Patients

The characteristics of the 13 patients examined are shown in Table I. All patients had both HIV and HCMV DNA in the peripheral blood mononuclear cells (2×10^5 PBMC) by the multiplex nested polymerase chain reaction (nPCR) described below. The total lymph node cells (2×10^5 cells) of the four lymph nodes included in this study were also PCR-positive for both viruses.

Preparation of Cells

Peripheral blood mononuclear cells (PBMC) were separated from EDTA blood by Ficoll gradient centrifugation and washed in phosphate-buffered saline (PBS). Lymph nodes were obtained by biopsy for diagnostic purposes and were disrupted mechanically. The cells were filtered (size of the pores: 100 μm) and then washed in PBS.

ACH-2 cells, containing one HIV genome copy per cell, were obtained from the American Type Culture Collection [Clouse et al., 1989a]. Cells were cultured in RPMI supplemented with 10% fetal calf serum (FCS). Human foreskin fibroblasts (HFF), infected with the Ad169 strain of HCMV, were cultured in Eagles' MEM supplemented with 5% FCS. Cells were passaged twice weekly and mixed with uninfected HFF at a ratio of 1:5.

Staining of Cells

Antibodies were obtained as follows: CD4: Leu-3a FITC, Becton Dickinson, Heidelberg, Germany (undiluted); CD14: IOM2 PE, Immunotech Dianova, Hamburg, Germany (undiluted). 10^6 PBMC from patients were suspended simultaneously in 20 μl anti-CD4 and in 20 μl anti-CD14 antibody and incubated 15 min on ice. Cells were washed three times in PBS. For FACS-sorting cells were then fixed in 1.5% paraformaldehyde in PBS for 30 min on ice and washed three times in PBS.

Cell Sorting by FACS

The use of an automated cell deposition unit (ACDU, Becton Dickinson, San Jose, CA) to sort defined cell numbers for the analysis by polymerase chain reaction (PCR) has been described in detail elsewhere [Bertram et al., 1995]. In brief, PCR reaction tubes (MicroAmp Reaction Tubes, Applied Biosystems, Weiterstadt, Germany) containing 25 μl lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 8.3) were prepared, and arranged in a rack (Micro Amp Retainer Set, Applied Biosystems). Cells were sorted directly into PCR tubes by a FACS-based ACDU. For each patient cells were sorted as follows:

$CD4^+$ $CD14^-$ lymphocytes:	2 samples containing	1000 cells each
	2 samples containing	500 cells each (patient 1–4)
		300 cells each (patient 5–13)
	6 samples containing	100 cells each
	12 samples containing	30 cells each
	12 samples containing	10 cells each
$CD14^+$ $CD4^{\pm}$ monocytes:	6 samples containing	1000 cells each
	6 samples containing	300 cells each
	12 samples containing	100 cells each
	12 samples containing	30 cells each

TABLE II. Primers Used for Detection of HIV and HCMV DNA[a]

Outer primers	Jam 17	5′-tac agg agc aga tga tac ag-3′
	Jam 20	5′-cct ggc ttt aat ttt act gg-3′
	CMV 17	5′-gac ccg ctg ttt cca gag ttg gcc gaa g-3′
	CMV 18	5′-gga gca ctg agg caa gtt ctg caa tgc cgc-3′
Inner primers	Jam 18	5′-gga aac caa aaa tga tag gg-3′
	Jam 19	5′-att atg ttg aca ggt gta gg-3′
	CMV 19	5′-ttt gaa cga gtg acc gag gat tgc aac gag-3′
	CMV 23	5′-gcc atc cac atc tcc cgc tta tcc tca gg-3′

[a]The primers for HIV-1 were from the gag/pol region, the primers for HCMV flanked the exon 3–4 splice junction of the HCMV IE1 gene.

The number of samples for each cell number is a minimum number. After FACS sorting, proteinase K was added (final concentration: 1.5 mg/ml). After an incubation of 2 hr at 60°C, the enzyme was inactivated for 10 min at 95°C.

Multiplex nPCR

A multiplex nested PCR (nPCR) detecting HIV and HCMV DNA with high sensitivity was carried out. As a control, a plasmid containing HIV DNA (bp224-5581 of HIV-1_{Bru}) and the plasmid pRR 47 [Stamminger et al., 1991] containing IE1 of HCMV were diluted in 25 μl PCR lysis buffer to three genome equivalents per control sample. To each sample 25 μl PCR mixture [50 mM KCl, 0.1% Triton® x-100, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 400 μM each of all four dNTPs], including 8 pmol each of the primers jam17 and jam20, 15 pmol of the primers CMV17 and CMV18, and 1.25 units of Taq polymerase (Promega, Madison, WI), were added. Amplification within 30 cycles was undertaken in a Gene Amp 9600 thermal cycler (Perkin Elmer, Norwalk, CT; 1 min at 95°C/1 min at 65°C for 2 cycles and 1 min at 58°C for the remaining cycles/1 min at 72°C). For the second round, 2 μl of first round amplication product was added to 25 μl of second round PCR mix [50 mM KCl, 0.1% Triton® x-100, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 200 μM each of all four dNTPs], including 8 pmol primers jam18 and jam19, 15 pmol of primers CMV19 and CMV23, and 1.25 units of Taq polymerase per sample. Amplification conditions were the same as in the first round. The primers used are shown in Table II. Amplification products were analysed by electrophoresis in a 2% agarose gel stained with 0.5 μg/ml ethidium bromide. The sizes of the amplification products were 462 bp for HCMV IE1 and 130 bp for HIV.

Statistics

The percentage of HIV- and HCMV-infected cells (in the sorted $CD4^+$ lymphocytes and $CD14^+$ monocytes) were determined from the number of DNA-positive samples using Poisson distribution:

$$1/n * \ln 1/(1-r) = p$$

p = Portion of infected cells, n = Number of sorted cells per PCR tube,
r = Number of PCR+ tubes/total number of tubes. If n = 1 the portion of infected cells was set equal to r.

RESULTS

PCR for the Simultaneous Detection of HCMV and HIV in Single Cells

Using a FACS-associated automated cell deposition unit (ACDU), defined cell numbers can be sorted into the wells of a microtiter plate. If PCR reaction tubes are arranged in a rack in microtiter form, cells can be sorted directly into the PCR tubes. This direct sorting was found to be important as single sorted cells could not be transferred reproducibly from microtiter plates to PCR tubes.

Several controls were carried out to evaluate the accuracy of cell deposition by ACDU and the sensitivity of PCR. Single ACH-2 cells, containing one HIV genome per cell, and single HCMV-infected human fibroblasts (HFF) were sorted and examined by nPCR. Of 228 single sorted ACH-2, 220 were positive for HIV DNA (96.5%). HCMV DNA was not amplified. Of 36 sorted HCMV-infected HFF, all were positive for HCMV DNA and HIV DNA was not amplified (data not shown). Furthermore, one ACH-2 and one HCMV-infected HFF each were sorted into 36 PCR tubes. The PCR was positive for HCMV as well as for HIV for all 36 samples (data not shown). As a further control, dilutions of the HIV-1_{Bru} plasmid and the HCMV pRR 47 plasmid were used. Samples containing ten and three copies of both plasmids could always be simultaneously detected. [For further description of the method used see Bertram et al., 1995.]

Analysis of Blood and Lymph Node Samples for Coinfection With HCMV and HIV

The number of HCMV-infected cells was reported to be below 0.1% in lymphocytes and below 1% in monocytes [Dankner et al., 1990]. The values published for HIV vary considerably depending on the method used [Embretson et al., 1993; Patterson et al., 1993; Re et al., 1994; Hufert et al., 1989; Schnittman et al., 1989]. To study coinfection, it is important to know the viral load for both viruses. If the level of infection for HCMV and HIV were high, coinfection of individual cells could be studied by examining a reasonable number of single cells. Low infection levels would require many single cell PCR reactions to detect potential coinfection, which is highly inefficient. Therefore, the first aim of the study was to determine the viral load for HIV and HCMV in the cell populations of interest.

Using ACDU and multiplex nested PCR, the viral load

TABLE III. Percentage of HIV- and HCMV-infected CD4+ Lymphocytes and CD14+ Monocytes[a]

		% infected			
		CD4+ lymphocytes		CD14+ monocytes	
	Patient	HIV	HCMV	HIV	HCMV
PBMC only	1	0.43	0.14	<0.05	0.58
	2	2.2	0.08	0.11	<0.05
	3	5.9	0.12	<0.05	<0.05
	4	0.99	0.05	<0.05	<0.05
	5	4.1	<0.05	<0.05	0.23
	6a	1.7	<0.05	<0.05	<0.05
	6b	2.1	<0.05	<0.05	0.11
	7	2.05	<0.05	<0.05	<0.05
	8	6.2	<0.05	<0.05	<0.05
	9	2.6	<0.05	<0.05	0.23
Lymph nodes and PBMC	10 LN	4.86	<0.05	nt	nt
	PBMC	1.7	<0.05	<0.05	<0.05
	11 LN	1.35	<0.05	nt	nt
	PBMC	0.93	<0.05	<0.05	<0.05
	12 LN	2.1	0.11	nt	nt
	PBMC	0.7	<0.05	nt	nt
	13 LN	1.45	<0.05	nt	nt
	PBMC	1.6	<0.05	nt	nt

[a]nt = not tested, LN = lymph node, PBMC = peripheral blood mononuclear cells. Patient 6 was examined at two different times.

for HIV and HCMV of human CD4+ lymphocytes in blood and lymph nodes and of CD14+ blood monocytes was determined. Between 10 and 1000 cells per sample were sorted by ACDU directly into PCR tubes and then examined for HCMV and HIV DNA using a highly sensitive multiplex nPCR. Similar to classical dilution studies, the percentage of infected cells was then calculated using the Poisson distribution (see Materials and Methods) [Bertram et al., 1995].

The viral loads are summarised in Table III. Between 0.43% and 6.2% of the blood CD4+ lymphocytes were infected with HIV. In lymph nodes the values were between 1.35% and 4.86%. The level of infection for HCMV in CD4+ lymphocytes was always found to be ≤0.14%. CD14+ monocytes could not be detected in lymph nodes, so they were only analysed in the blood. The percentage of HIV-infected monocytes was always ≤0.11%. The values for HCMV were also ≤0.11% in most patients. Higher infection levels were only found in three individuals, all with manifest HCMV disease. The first patient suffered from HCMV retinitis (0.58% infected monocytes). For patient no. 5, suffering from HCMV gastritis, 0.23% infected monocytes were found. For patient no. 9, with an encephalitis, the value was 0.23%.

Based on the low viral load for HCMV in CD4+ cells and for both viruses in CD14+ monocytes we did not expect frequent coinfection of individual leukocytes. In fact, both viral genomes were present simultaneously only in the CD4+ lymphocyte population (patients no. 1 to 4). In these patients HCMV and HIV DNA were simultaneously detected in seven samples. The number of cells sorted for these samples and the number of HIV-positive, HCMV-positive, and double-positive samples are shown in Table IV. The number of double-positive samples was not higher than expected statistically, which excludes the likelihood that a significant portion of individual cells harboured both viruses.

TABLE IV. Detection of Viral DNA in Multiple Samples Containing Defined Numbers of FACS-Sorted CD4+ Lymphocytes[a]

Patient no.	CD4+ lymphocytes per sample	HIV+	HCMV+	Double+
1	500	1/2	1/2	1/2
2	50	27/36	2/36	2/36
3	10	38/84	1/84	1/84
4	1000	7/8	3/8	3/8

[a]Only the results for the cell numbers, for which double-positive samples were found, are included.

In lymph nodes of 144 samples with between 10 and 1000 cells were examined. Only one sample was HCMV DNA-positive and 59 samples were positive for HIV DNA. None of the samples were double-positive. Coinfection of individuals cells was thus found to be rare.

DISCUSSION

In vitro studies have shown that HIV-1 gene expression and replication are stimulated by coinfection with HCMV. However, our data demonstrate that transactivation most likely does not influence the viral load in blood and lymphatic tissue in vivo. The level of infection with HCMV was low and double-infections of individual cells with both viruses were found to be rare in vivo.

HCMV immediate early gene products have been shown to transactivate the HIV LTR [Biegalke and Geballe, 1991; Walker et al., 1992]. However, if such a

transactivation is to be of any clinical significance both viruses would have to be present within the same cell in vivo. Coinfection occurs most likely by chance. If x% of the cells are infected with HIV and y% with HCMV, 1/100 * (x*y) % of the cells are expected to be coinfected. This means that significant coinfection only occurs when the viral load for both of the viruses is high. We show, however, that the viral load in human leukocytes especially for HCMV is very low. The values for HIV ranged between 0.46% and 6.2% infected cells in $CD4^+$ lymphocytes from blood and lymph nodes. The level of HCMV infection in $CD4^+$ lymphocytes was always found to be $\leq$0.14% i.e., less than one in 100 HIV-infected $CD4^+$ cells would statistically be expected to also harbour HCMV and that less than one in 10^3 total $CD4^+$ lymphocytes would be coinfected. The percentage of HIV-infected monocytes was always $\leq$0.11% and the level of HCMV infection under 1%. Consequently coinfection of these cells would be expected to occur in less than one of 10^5 monocytes. Thus, the predicted level statistically of coinfection in blood and lymph nodes in vivo would be too low to influence the viral load for HIV simply via direct transactivation of HIV gene expression by HCMV gene products in coinfected cells.

The possibility, however, must be considered that the infection with one virus could increase the probability for infection with the other. Coinfection would then occur more frequently than expected statistically. In vitro, such an enhancement has been described. McKeating et al. [1990] have shown that Fc receptor-like proteins on the surface of HCMV-infected fibroblasts allow the uptake of HIV into these cells, which are not otherwise permissive for HIV. Our data, however, do not provide evidence for such an enhancing effect in leukocytes. Altogether only seven of over 1000 examined blood samples and none of 144 samples from lymph nodes were found double-positive for HIV and HCMV DNA. For all seven samples the frequency of double-positive samples was not higher than expected statistically (from the number of samples sorted, the number of cells per sample, and the percentage of infected cells). Thus, the two viruses in these samples were most likely in different cells. Therefore, as expected statistically, coinfection of individual leukocytes with HCMV and HIV in vivo was found to be a very rare event and the intracellular interactions of HIV and HCMV observed in vitro may not explain the more rapid progression to overt AIDS in HCMV-seropositive individuals.

On the other hand, coinfection of brain and retina cells with HIV and HCMV have been described in AIDS patients [Nelson et al., 1988; Rummelt et al., 1994]. Our results clearly do not question the possibility that HCMV in the brain or retina may contribute locally to HIV-associated pathology by mechanisms such as enhancement of HIV entry into cells and stimulation of HIV replication. The major target cell for HIV, however, is the $CD4^+$ lymphocyte in blood and lymph nodes. A local direct effect of HCMV in the brain of HIV replication is, therefore, not expected to influence immune depletion and disease progression in HIV-infected patients.

HIV p24 antigen has been detected in cytomegalic cells in the lung of two AIDS patients with disseminated HCMV infection [Finkle et al., 1991]. This may reflect productive coinfection of these cells with both viruses in the lung or phagocytosis of viral antigens. Coinfection in the lung could indeed influence viral load and disease progression. This holds true, if the lung was an important site of HIV-1 replication in the asymptomatic phase of HIV-infection. However, little is known about the lung as a viral reservoir for HIV-1. Further studies must clarify this issue.

The results suggest that HCMV must act by some indirect mechanism such as the activation of cytokine production. Supernatants from lipopolysaccharide-stimulated monocytes have been shown to increase HIV expression in chronically infected cell lines [Clouse et al., 1989a]. Tumor necrosis factor alpha (TNF-α) most likely mediates this effect [Clouse et al., 1989b]. HCMV-stimulated PBMC were also capable of inducing HIV-1 replication in co-culture with HIV-infected PBMC. Monoclonal antibodies to TNF-α blocked this activity [Peterson et al., 1992]. Thus TNF-α may play a key role in the HCMV-induced acceleration of disease progression in HIV-1-infected patients. Furthermore, HCMV alone causes immunosuppression with a depressed lymphocyte response to T-cell as well as a reduced natural killer cell activity [Schrier et al., 1986]. Therefore, infections with HIV and HCMV may co-operatively contribute to the progressive loss of immune function. In the future, it will be necessary to study such indirect mechanisms of interaction between HIV and HCMV more closely.

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